Modulation of antagonist binding to histamine H₁-receptors by sodium ions and by 2-amino-2-hydroxymethyl-propan-1,3-diol HCl

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1 NaCl (100 mM) reduced the potency of (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) as an inhibitor of the binding of [³H]-mepyramine to histamine H₁-receptors on guinea-pig cerebellar membranes to a greater extent than that of mepyramine, consistent with the greater inhibitory effect of Na⁺ on the binding of [³H]-QMDP than on the binding of [³H]-mepyramine.

2 The concentration of 2-amino-2-hydroxymethyl-propan-1,3-diol HCl (Tris, HCl) buffer, pH 7.5, present had little effect on the temelastine-insensitive binding of [³H]-mepyramine, but caused a concentration-dependent inhibition of the binding of [³H]-mepyramine sensitive to 1 μM temelastine (H₁-receptor binding), with an approximate IC₅₀ of 75 mM, assuming that complete inhibition would have been achieved.

3 Inhibition of [³H]-mepyramine binding by Na⁺ was more marked in 10 mM than in 50 mM Tris HCl and was not evident in 200 mM Tris HCl.

4 The K_d for the temelastine-sensitive binding of [³H]-mepyramine measured in 10 mM Tris HCl, 0.24 ± 0.01 nM, was increased by 2.2 ± 0.2 fold by 100 mM NaCl, without any significant change in the maximum binding (B_{max}). The B_{max} for [³H]-mepyramine was similarly unchanged in 50 mM Tris HCl, but the K_d was increased 2.5 ± 0.2 fold.

5 The K_d for the temelastine-sensitive binding of [³H]-mepyramine was also increased in 50 mM, compared with 10 mM, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] KOH (HEPES.KOH) buffer (K_d 0.25 ± 0.02 nM in 10 mM HEPES), but the evidence for an interaction between HEPES and Na⁺ was less clear.

6 The effect of 100 mM NaCl on the inhibition of [³H]-mepyramine binding in 10 mM Tris HCl was examined for a range of antagonists. The decrease in potency caused by Na⁺ was greatest for triprolidine, (+)-chlorpheniramine and benzylcholine (9.6–10.3 fold increase in K_d values) but the binding of mepyramine and promethazine was much less affected (1.8 and 1.9 fold increase in K_d respectively). The K_d for temelastine was not significantly changed. In contrast to the general decrease in antagonist affinity in the presence of Na⁺, the K_d for MDL 16,455A (4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]-α,α-dimethylbenzene acetic acid) was increased, but only by 1.5 fold.

7 It is concluded that Na⁺ can act as an allosteric effector of the binding of antagonists at the histamine H₁-receptor. Tris HCl also appears to have an allosteric action at the H₁-receptor.

Keywords: Histamine H₁-receptors; [³H]-mepyramine; sodium ions; Tris.HCl buffer; HEPES.KOH buffer; temelastine; terfenadine; MDL 16,455A; picumast

Introduction

G-protein-coupled receptors possess a highly conserved aspartate residue in the second transmembrane loop which appears to be associated with the modulation of agonist binding by intracellular Na⁺ and H⁺ (for a review see Hibert *et al.*, 1993). The most direct evidence for the involvement of this residue comes from mutation studies with the α₂-adrenoceptor (Horstman *et al.*, 1990) and the dopamine D₂-receptor (Neve *et al.*, 1991), in which substitution of the aspartate with asparagine (α₂-receptor) or alanine (D₂-receptor) abolished the regulatory action of Na⁺ on ligand binding. With the histamine H₁-receptor the regulation of agonist binding by Na⁺ (Chang & Snyder, 1980) presumably involves Asp-74 in the second transmembrane loop of the H₁-receptor cloned from bovine adrenal medulla (Yamashita *et al.*, 1991) or Asp-73 in the rat H₁-receptor (Fujimoto *et al.*, 1993). However, antagonist binding can also be modulated by Na⁺, as originally demonstrated for opiate receptors (Pert & Snyder, 1974; Paterson *et al.*, 1986), and we have reported that Na⁺ ions distinguish between the binding of two antagonist radioligands to the histamine H₁-receptor (Treherne *et al.*, 1991). We describe here a study of the action of Na⁺ on the binding

of a wider range of antagonists to the H₁-receptor, as reflected by the effect of Na⁺ on antagonist potency in inhibiting the binding of [³H]-mepyramine to guinea-pig cerebellar membranes. At the same time, in view of the reported effect of the 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) cation in obscuring the modulation by Na⁺ of [³H]-nitrendipine binding to L-type Ca²⁺ channels (Bolger & Skolnick, 1986), we have investigated the possible modulation of H₁-antagonist binding by Tris HCl buffers.

Methods

Preparation of cerebellar membranes

Cerebella from Dunkin-Hartley strain guinea-pigs (males, 250–600 g; Tucks, Battlebridge, Essex) were washed in 10 or 50 mM 2-amino-2-hydroxymethylpropan-1,3-diol hydrochloride (Tris-HCl) buffer, pH 7.5, or 10 or 50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES) buffer, pH 7.5, containing 1 mM ethylenediaminetetracetic acid (EDTA), and then homogenized in 10 volumes of the same buffer in a teflon-glass homogeniser with a motor-driven pestle (3,000 r.p.m., 5 or 6 up and down strokes). The

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homogenate was centrifuged at 17,000 g for 30 min and the pellet then resuspended in buffer but without EDTA and centrifuged again at 17,000 g for 30 min. The resulting pellet was suspended in 10 or 50 mM HEPES or Tris buffer, pH 7.5, to give a final protein concentration of 6–10 mg ml⁻¹ and was stored at -20°C until required for use. Protein was determined essentially as described by Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Measurement of the binding of [³H]-mepyramine

Incubations in 10 or 50 mM HEPES or Tris-HCl buffer, pH 7.5, contained [³H]-mepyramine (usually in the concentration range 0.4–0.9 nM for inhibition curves), inhibitor, 100 mM NaCl (where appropriate) and cerebellar homogenate (0.19–0.27 mg protein) in a total volume of 1.00–1.11 ml (four replicates at each inhibitor concentration, 8–16 replicates in the absence of inhibitor, spread through the experiment). For a given inhibitor, curves in the presence and absence of NaCl were always determined in the same experiment. HEPES buffers were adjusted to pH 7.5 with KOH, giving approximately 36 mM K⁺ in 50 mM HEPES buffer. In experiments in which H₁-receptor binding was measured as a function of the concentration of [³H]-mepyramine, non-specific binding was determined as the binding insensitive to inhibition by 1 µM temelastine, which has a low affinity for secondary, non-H₁-receptor, binding sites for [³H]-mepyramine (Arias-Montaña & Young, 1993a). The extent of the non-specific binding of 0.4–0.6 nM [³H]-mepyramine in 10 or 50 mM Tris-HCl buffer was usually 9–16% of total binding. Equilibration was for 60 min at 30°C and was terminated by filtration through Whatman GF/B glass fibre paper, pre-soaked in 0.3% (w/v) polyethylenimine for 3–5 h, using a Brandel (Gaithersburg, Md, U.S.A.) cell harvester. The filters were washed with ice-cold buffer and then transferred to scintillation insert vials containing 4.0 ml scintillator (Emulsifier-Safe, Packard) and allowed to stand at room temperature at least 2 h before determination of tritium by liquid scintillation counting.

Analysis of data

Curves of inhibition of [³H]-mepyramine binding were fitted to the equation: % of uninhibited binding of [³H]-mepyramine = (100-NS)/((A/IC₅₀)ⁿ + 1) + NS where n is the Hill coefficient, A is the concentration of inhibitor, IC₅₀ is the concentration of inhibitor required for 50% inhibition of the binding of [³H]-mepyramine sensitive to the inhibitor, and NS is the percentage of the binding insensitive to the inhibitor, using the Harwell Library non-linear regression routine VB01A. All points were weighted according to the reciprocal of the approximate variance associated with them. Where n did not differ significantly from unity the dissociation constant of the inhibitor was calculated from the relationship: K_d = IC₅₀/([³H]-mepyramine)K_{d(mepyramine)} + 1. In the special case of mepyramine versus [³H]-mepyramine this simplifies to K_d = IC₅₀ - [³H]-mepyramine, assuming that the substitution of one atom of tritium for one atom of hydrogen has no significant effect on the affinity of mepyramine.

Curves of the temelastine-sensitive binding of [³H]-mepyramine versus the concentration of [³H]-mepyramine were fitted as above to the equation:

$$[{}^3\text{H}]\text{-mepyramine bound} = B_{\text{max}} \cdot C^n / (C^n + EC_{50}^n)$$

where C is the concentration of radioligand, EC₅₀ is the concentration giving half-maximal binding, n is the Hill coefficient and B_{max} is the maximum binding. Where the Hill coefficient was not significantly different from unity (in all experiments except one with 10 and 50 mM HEPES), the data were fitted to a hyperbola (n = 1 in the equation above) to obtain the K_d (= EC₅₀) and B_{max}.

The overall value of K_d from a number of determinations was calculated as the weighted mean ± s.e.mean from indi-

vidual values and their estimated standard errors, derived from the best-fit estimates of IC₅₀. For the three incubation conditions with the largest number of determinations, the K_d of mepyramine in 10 and 50 mM Tris HCl and in 10 mM HEPES KOH, the final estimate was similar when the weighted mean was calculated from values of log(K_d) and the approximate standard error, i.e. 0.24 ± 0.01 and 0.25 ± 0.01 nM in 10 mM Tris, 0.77 ± 0.03 and 0.80 ± 0.03 nM in 50 mM Tris and 0.25 ± 0.02 and 0.29 ± 0.02 nM in 10 mM HEPES, using K_d and log (K_d), respectively.

Statistical comparison of parameters characterizing concentration-response curves in 10 and 50 mM Tris or HEPES buffer was made by fitting the curves simultaneously (using the NAG library routine E04FDF) and assessing the increase in the residual sum of squares when a parameter was constrained to be the same for both curves, by calculating the F-statistic (Rodbard, 1974):

Figure 1 Effect of 100 mM NaCl on the inhibition of [³H]-mepyramine binding in 50 mM Tris HCl buffer by (a) (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) and (b) mepyramine. Values are means ± approximate s.e.mean of quadruplicate determinations from single experiments with (a) 0.63 nM and (b) 0.77 nM [³H]-mepyramine. Where no error bars are shown the error was within the size of the symbol. The curves drawn are the weighted best-fit lines to a Hill equation (see Methods). Best-fit values: (a) Hill coefficient (n_H) 1.03 ± 0.11 and 0.92 ± 0.03, IC₅₀ 1.1 ± 0.1 and 5.0 ± 0.2 nM; (b) n_H 0.98 ± 0.04 and 1.03 ± 0.05, IC₅₀ 1.6 ± 0.1 and 1.8 ± 0.1 nM, in the absence and presence of 100 mM NaCl, respectively. In the presence of NaCl the specific binding of [³H]-mepyramine was reduced by 24% in (a) and 21% in (b). The experiment with (+)-QMDP was repeated twice more and that with mepyramine three times more, with similar results. (○) No NaCl, (●) 100 mM NaCl present.

$$F = \{ (SS_2 - SS_1) / (d.f._2 - d.f._1) \} / (SS_1 / d.f._1)$$

where SS_2 is the sum of squares when a parameter is shared ($d.f._2$ degrees of freedom) and SS_1 the sum of squares when all parameters are allowed to float freely for each curve ($d.f._1$ degrees of freedom).

Drugs

[3H]-mepyramine (21 and 26 Ci mmol $^{-1}$) was obtained from Amersham International. Histamine dihydrochloride, mepyramine maleate and promethazine hydrochloride were purchased from Sigma. (+) and (–)-Chlorpheniramine maleate were gifts from Schering, hydroxyzine hydrochloride from Pfizer, icotidine trihydrochloride and temelastine from Smith, Kline and French Research, terfenadine and MDL 16,455A hydrochloride (4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]- α,α -dimethylbenzene acetic acid) from Marion Merrell Dow, tiagabine hydrochloride from Novo Nordisk and picumast dihydrochloride from Boehringer Mannheim. N-methyl-4-methyldiphenhydramine iodide [(+)-QMDP], pirdonium bromide and norpirdonium maleate were kindly provided by Prof. H. Timmerman, Vrije Universiteit, Amsterdam and triprolidine hydrochloride by Dr A.F. Casy, University of Bath. Benzilylcholine chloride was synthesized by Mr B. Peck.

Results

Effect of Na $^+$ on antagonist inhibition of [3H]-mepyramine binding in 50 mM Tris HCl

Consistent with the effect of Na $^+$ in causing a greater inhibition of the binding of [3H]-mepyramine to H $_1$ -receptors in guinea-pig cerebellar membranes than that of [3H]-QMDP (Treherne *et al.*, 1991), 100 mM NaCl had a greater effect on the position of the curve of (+)-QMDP inhibition of [3H]-mepyramine binding to cerebellar membranes, measured in 50 mM Tris HCl buffer, than on that of mepyramine (Figure 1). The very small effect of Na $^+$ on the position of the curve of mepyramine inhibition of [3H]-mepyramine binding, indicates that there is only a small change in the K_d for mepyramine, since the concentration of the radioligand was the same throughout the experiment. The ratios of the K_d s in the presence and absence of Na $^+$ are set out in Table 1, together with the values for temelastine and histamine. The extent of the increase in the apparent K_d for the agonist histamine produced by 100 mM NaCl in 50 mM Tris HCl, 10.4 fold (Table 1), was similar to that, 9.8 fold, reported by Chang & Snyder (1980) using the same buffer.

Effect of Tris HCl buffer concentration on [3H]-mepyramine binding and the modulatory action of Na $^+$

The initial choice of 50 mM Tris HCl buffer for our studies (Treherne *et al.*, 1991) was made for compatibility with earlier measurements of the effects of cations on H $_1$ -agonist inhibition of [3H]-mepyramine binding (Chang & Snyder, 1980). However, Tris HCl itself proved to be an effective inhibitor of [3H]-mepyramine binding to cerebellar membranes (Figure 2). There was little effect on the temelastine-insensitive binding of [3H]-mepyramine, but the temelastine-sensitive binding (H $_1$ -receptor binding) was markedly reduced as the buffer strength was increased, with an IC $_{50}$ of approximately 75 mM Tris HCl, assuming that all of the temelastine-binding would have been inhibited (Figure 2). The pH was 7.5 at 30°C throughout.

The concentration of Tris HCl present had a marked effect on the modulation of [3H]-mepyramine binding by Na $^+$. In 10 mM Tris HCl, [3H]-mepyramine binding was inhibited more strongly by Na $^+$ than in 50 mM Tris HCl and in 200 mM Tris HCl the inhibitory action of Na $^+$ was abolished

Figure 2 Inhibition of [3H]-mepyramine binding by Tris HCl. Values are means $\pm$ s.e.mean of the binding of 0.35 nM [3H]-mepyramine from quadruplicate determinations within a single experiment. Error bars were within the size of the symbols. The cerebellar homogenate used in these experiments was prepared in 10 mM Tris HCl. The whole experiment was repeated twice more with similar results. (○) Total binding; (●) binding in the presence of 1 μ M temelastine.

Figure 3 Effect of the concentration of Tris HCl buffer present on the inhibition of the H $_1$ -receptor binding of [3H]-mepyramine by NaCl. Values are means $\pm$ s.e.mean of quadruplicate measurements within a single experiment of the difference of the binding of 0.52 nM [3H]-mepyramine in the absence and presence of 1 μ M temelastine. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated twice more with similar results. (●) 10 mM Tris; (○) 50 mM Tris; (■) 200 mM Tris.

(Figure 3). The implication is that Na $^+$ and Tris are both allosteric inhibitors of [3H]-mepyramine binding. However, it is not clear that they are acting by a common mechanism, since the shift of the inhibition curve for histamine in 50 mM Tris HCl was as great as in 10 mM Tris HCl (Table 1).

The interaction of both Na $^+$ and Tris HCl with the H $_1$ -receptor appeared to be reversible, since the effect of 100 mM NaCl on the saturation binding of [3H]-mepyramine to the H $_1$ -receptor measured in 10 mM Tris HCl was to cause a decrease in the affinity without any significant effect on the maximum binding (Figure 4a). The mean increase in the K_d

Figure 4 Binding of [³H]-mepyramine to histamine H₁-receptors (a) in 10 mM Tris HCl in the presence and absence of 100 mM NaCl and (b) in 10 and 50 mM Tris HCl buffers. Values are the means $\pm$ s.e.mean from quadruplicate determinations within a single experiment of the difference in the binding of [³H]-mepyramine to cerebellar membranes in the absence and presence of 1 μ M temelastine. Where no error bars are shown the error was within the size of the symbol. The curves drawn are the weighted best-fit lines to a Hill equation (see Methods). Best-fit values: (a) Hill coefficient (n_H) 0.90 ± 0.10 and 0.99 ± 0.09 ; EC_{50} 0.26 ± 0.06 and 0.62 ± 0.15 nM, in the absence and presence of 100 mM NaCl, respectively; (b) n_H 1.14 ± 0.14 and 0.81 ± 0.09 ; EC_{50} 0.21 ± 0.03 and 0.99 ± 0.29 nM, in 10 and 50 mM Tris, respectively. Each experiment was repeated twice more with similar results. (a) (○) 10 mM Tris HCl; (●) 10 mM Tris HCl + 100 mM NaCl (b) (○) 10 mM Tris HCl; (●) 50 mM Tris HCl.

was by a factor of 2.2 ± 0.2 (three determinations). Similarly, the binding curve for [³H]-mepyramine to 50 mM Tris HCl was shifted to lower affinity compared with that in 10 mM Tris HCl, but again without any significant effect on the maximum binding (Figure 4b). There were no consistent or significant differences in the best-fit Hill coefficients for the pairs of curves in either set of experiments. The mean increase in the K_d in 50 mM Tris was 2.5 ± 0.2 fold (3).

The overall mean K_d for [³H]-mepyramine in 10 mM Tris HCl, taken from saturation binding and inhibition curves, was 0.24 ± 0.01 nM (eight determinations) and in 50 mM Tris HCl 0.77 ± 0.03 nM (7).

Effect of HEPES buffer on [³H]-mepyramine binding

Increasing the concentration of HEPES KOH buffer from 25 to 50 mM caused an inhibition of [³H]-mepyramine binding

(Figure 5). K⁺ is a relatively weak inhibitor of the binding of both [³H]-mepyramine and [³H]-QMDP (Treherne *et al.*, 1991), but the presence of an appreciable concentration of K⁺, circa 36 mM in 50 mM HEPES KOH, potentially complicates the interpretation of the data and we have not examined higher HEPES KOH buffer concentrations. The amount of K⁺ present was adjusted with KCl to be the same as in the 50 mM buffer throughout the experiment.

A possible interaction between HEPES and Na⁺ was less clear (Figure 5) than in Tris HCl. The percentage inhibition of [³H]-mepyramine binding by 100 mM NaCl was consistently greater in 50 mM than 10 mM HEPES ($13 \pm 1\%$ and $23 \pm 2\%$ inhibition in 10 and 50 mM HEPES, respectively, 5 paired determinations), but the effect of NaCl appeared to be to reduce the temelastine-sensitive binding of [³H]-mepyramine by a constant absolute amount (Figure 5).

The H₁-receptor binding of [³H]-mepyramine in 50 mM HEPES KOH was shifted to lower affinity compared with the saturation binding in 10 mM HEPES KOH, in an analogous fashion to that observed in 10 and 50 mM Tris HCl (cf. Figure 4), without consistent effect on the maximum binding. The mean K_d for [³H]-mepyramine in 50 mM HEPES was 1.0 ± 0.1 nM (3) and that in 10 mM HEPES, calculated from both saturation binding and from inhibition curves, was 0.25 ± 0.02 nM (6).

Dependence of the direction and magnitude of Na⁺ shifts of inhibition curves on antagonist structure

The inhibition of [³H]-mepyramine binding in 10 mM Tris HCl and 10 mM HEPES KOH appears to be minimal (Figures 2 and 5) and shifts in the curves of inhibition of [³H]-mepyramine binding by mepyramine, (+)-QMDP, temelastine and histamine were similar in the two buffers. The ratios of the K_d values in the presence and absence of 100 mM NaCl, derived from the inhibition curves, are given in Table 1. The ratios for mepyramine and (+)-QMDP were greater in the 10 mM buffers than in 50 mM Tris HCl, but the difference was not statistically significant for mepyramine in 10 mM Tris HCl (as assessed by applying the Student-Newman-Keuls multiple range test to the weighted means derived

Figure 5 Inhibition of the histamine H₁-receptor binding of [³H]-mepyramine by HEPES KOH in the presence and absence of 100 mM Na⁺. The amount of K⁺ present was adjusted to be the same throughout the experiment by addition of the appropriate amount of KCl to incubations in which [HEPES] was less than 50 mM. Values are means $\pm$ s.e.mean from quadruplicate determinations within a single experiment of the binding of 0.59 nM [³H]-mepyramine sensitive to inhibition by 1 μ M temelastine. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated twice more with similar results. (○) HEPES alone; (●) HEPES + 100 mM NaCl.

Figure 6 Structures of some of the antagonists examined.

from ratios $\pm$ approximate s.e.). In contrast, for histamine the ratio of apparent K_d values did not differ significantly in any of the buffers (Table 1). The Hill coefficients for the histamine inhibition curves were similar in the three buffers and in the presence and absence of 100 mM NaCl (values given in the legend to Tables 1 and 2). Temelastine was little affected by Na⁺ in either 50 or 10 mM buffer (Table 1).

For the survey of the effect of Na⁺ on a wider range of antagonists, measurements were made in 10 mM Tris HCl to avoid any possible complications introduced by the presence of K⁺ in the buffer. The changes in affinity produced by 100 mM NaCl, expressed as the ratio of K_d values in the presence and absence of Na⁺ are given in Table 2. The largest decreases in affinity, approximately 10 fold, were observed with benzylylcholine (primarily a muscarinic antagonist), (+)-chlorpheniramine and triprolidine. The K_d for temelastine was not significantly changed. However, for one of the antagonists, MDL 16,455A (Figure 6) the effect of Na⁺ was to produce a significant increase in affinity, ratio of K_d values in the presence and absence of Na⁺ 0.67 ± 0.04 ($P < 0.05$, assuming the ratios to be normally distributed), but only by a factor of 1.5.

Discussion

It is clear from the data presented above that the modulatory action of Na⁺ ions on ligand binding to the histamine H₁-receptor is not confined to agonists, as originally proposed (Chang & Snyder, 1980), but also affects the binding of antagonists in a direction and to an extent dependent on their structure. This is consistent with Na⁺ acting directly at the receptor as an allosteric regulator of ligand binding in a way well established for a number of G-protein-coupled receptors (Hibert *et al.*, 1993). The enhancement of the binding of at least one of the antagonists studied is strong evidence that Na⁺ does induce a conformation change. It is

Table 1 Influence of buffer composition on the effect of 100 mM NaCl on K_d values of H₁-receptor ligands

Inhibitor	$(K_d \text{ in presence of Na}^+) / (K_d \text{ in absence of Na}^+)$ measured in		
	50 mM Tris	10 mM Tris	10 mM HEPES
Mepyramine	1.1 ± 0.3 (4)	1.8 ± 0.2 (3)	2.5 ± 0.2 (3)
(+)-QMDP	4.0 ± 0.2 (3)	7.4 ± 0.5 (3)	9.4 ± 0.5 (3)
Temelastine	1.0 ± 0.1 (3)	0.9 ± 0.1 (3)	1.0 ± 0.1 (3)
Histamine	11.0 ± 0.5 (4)	10.0 ± 0.4 (3)	12.4 ± 0.8 (3)

Values of the ratios of K_d values are weighed means $\pm$ s.e.mean calculated from the ratios $\pm$ approximate s.e. of the best-fit values of the IC₅₀ for the inhibition of the inhibitor-sensitive binding of 0.4–0.9 nM [³H]-mepyramine in the presence and absence of 100 mM NaCl. The number of paired determinations of IC₅₀ $\pm$ Na⁺ is given in parentheses. Weighted mean K_d values in 50 mM Tris HCl, calculated from the best-fit IC₅₀ values in the absence of Na⁺: mepyramine 0.92 ± 0.02 nM, (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) 0.76 ± 0.03 nM, temelastine 0.28 ± 0.01 nM and histamine (apparent K_d mean Hill coefficient 0.77 ± 0.02) $1,970 \pm 50$ nM. The mean Hill coefficient for histamine in 50 mM Tris HCl in the presence of Na⁺ was 0.78 ± 0.02 . K_d values in 10 mM HEPES KOH: mepyramine 0.27 ± 0.04 nM, (+)-QMDP 0.20 ± 0.01 nM, temelastine 0.26 ± 0.02 nM, histamine (apparent K_d), $1,550 \pm 40$ nM. The Hill coefficients for histamine inhibition curves in 10 mM HEPES KOH were 0.74 ± 0.02 and 0.77 ± 0.03 in the absence and presence of Na⁺, respectively. K_d values in 10 mM Tris HCl are given in Table 2.

also clear, at least as far as antagonist binding is concerned, that the action of Na⁺ can be obscured in the presence of a sufficient concentration of Tris HCl buffer. The simplest explanation is that Tris HCl is also an allosteric effector at the H₁-receptor, although inhibition of [³H]-mepyramine binding is the only effect that we have demonstrated for Tris HCl acting alone. However, the antagonism between Na⁺

Table 2 Effect of 100 mM NaCl on the affinity of antagonists for the histamine H₁-receptor in cerebellar membranes

Antagonist	K_d (nM)	$(K_d + Na^+)/ (K_d \text{ no } Na^+)$
Benzylcholine	830 ± 10	10.3 ± 0.3 (3)
(+)-Chlorpheniramine	0.12 ± 0.01	10.2 ± 0.6 (3)
Triprolidine	0.05 ± 0.01	9.6 ± 0.4 (3)
(-)-Chlorpheniramine	34 ± 1	8.7 ± 0.3 (3)
(+)-QMDP	0.24 ± 0.01	7.4 ± 0.5 (3)
Norpirdonium	0.43 ± 0.02	5.6 ± 0.2 (3)
Tiagabine	3,260 ± 170	3.3 ± 0.3 (2)
Pirdonium	0.36 ± 0.01	2.9 ± 0.1 (4)
Picumast	0.25 ± 0.30	2.5 ± 0.2 (3)
Hydroxyzine	1.0 ± 0.1	2.0 ± 0.3 (3)
Promethazine	0.36 ± 0.01	1.9 ± 0.1 (3)
Mepyramine	0.36 ± 0.03	1.8 ± 0.2 (3)
Temelastine	0.36 ± 0.02	0.9 ± 0.1 (3)
Icotidine	7,140 ± 200	0.9 ± 0.1 (3)
Terfenadine	4.3 ± 0.2	0.8 ± 0.1 (3)
MDL 16,455A	33 ± 2	0.7 ± 0.1 (3)
Agonist		
Histamine	(1,520 ± 50)*	10.0 ± 0.4 (3)

Measurements of the inhibition of the binding of 0.42–0.92 nM [³H]-mepyramine to cerebellar membranes in the presence and absence of 100 mM NaCl were made in 10 mM Tris HCl buffer, pH 7.5. K_d values are weighted means ± s.e. mean calculated from the best-fit values of IC_{50} ± estimated error (see Methods) for inhibition curves in the absence of NaCl, taking the value of K_d for [³H]-mepyramine to be 0.36 nM, as determined from mepyramine inhibition curves. The values of the ratio of K_d values in the presence and absence of Na⁺ are the weighted means of the ratios from individual experiments, in which paired measurements were made of inhibition curves in the presence and absence of 100 mM NaCl (see legend to Table 1). There were no consistent changes in the Hill coefficient in the presence of Na⁺ and no consistent divergence of Hill coefficients from unity.

*Apparent K_d . The weighted mean values of the Hill coefficients for histamine were 0.74 ± 0.02 and 0.74 ± 0.03 in the presence and absence of 100 mM NaCl, respectively.

and Tris HCl is clear, since in 10 mM Tris HCl, Na⁺ inhibits [³H]-mepyramine binding at concentrations above 20 mM, whereas in 200 mM Tris HCl there is no effect of Na⁺ up to 100 mM (Figure 3). Were Tris HCl acting as a simple competitive antagonist at the H₁-receptor, then it would not be expected that the inhibitory action of Na⁺ would be abolished at a concentration of Tris HCl that does not cause complete inhibition of [³H]-mepyramine binding. The same antagonism between Tris HCl and Na⁺ is apparent in the increased effect of Na⁺ on the K_d for (+)-QMDP as the concentration of Tris HCl buffer is reduced from 50 mM to 10 mM (Table 1).

Both Na⁺ and Tris HCl appear to be reversible allosteric inhibitors of [³H]-mepyramine binding, since they both displace the K_d for [³H]-mepyramine binding curves to higher concentrations, without any significant change in B_{max} (Figure 4). However, there is some indication that the mechanisms of interaction of Na⁺ and Tris HCl with the H₁-receptor are not identical, since the extent of the decrease in the apparent K_d for histamine produced by 100 mM NaCl is not significantly different in 10 and 50 mM Tris HCl buffers (Table 1). Complexity of the conformational equilibria involved is further suggested by the observation that Li⁺, which is also reported to inhibit the binding of histamine (Chang & Snyder, 1980), inhibits equally the binding of [³H]-mepyramine and [³H]-QMDP (Treherne *et al.*, 1991). Which Tris species, protonated or uncharged, is involved in this interaction is unknown, although it would seem most likely to be the Tris cation.

The action of Tris HCl in obscuring the inhibitory effect of Na⁺ is one reason why the effect on antagonist binding was not more apparent in the original study of the effect of cations on [³H]-mepyramine binding in which measurements were made in 50 mM Tris HCl (Chang & Snyder, 1980), although the K_d for triprolidine was in fact decreased by 100 mM NaCl by a factor of nearly four. This underlines the dependence of dissociation constants determined from inhibition of radioligand binding to membrane preparations on the assay conditions employed. The same point has been made with some force for the determination of the relative affinities of antagonists for muscarinic receptor subtypes (Pedder *et al.*, 1991). It also emphasises the fundamental problem in-

herent in the use of membrane preparations in binding assays, in that no medium can mimic the physiological situation with intact cells, in which the cytoplasmic and extracellular faces of the receptor are exposed to solutions of very different ionic composition.

Na⁺ and Tris HCl are not the only agents which act as modulators of ligand binding to histamine H₁-receptors. We have recently demonstrated that Ni²⁺ is an allosteric inhibitor of [³H]-mepyramine binding to a membrane preparation from guinea-pig cerebral cortex (Arias-Montaña & Young, 1993b). The approach we adopted there to demonstrate the allosteric mechanism of action, namely the simultaneous analysis of a family of Ni²⁺ inhibition curves measured in the presence of a range of concentrations of [³H]-mepyramine, is not so easily applicable to Na⁺, since the inhibition curves with Na⁺ give no indication of having reached a level of maximum inhibition even at 200 mM. Whether Ni²⁺ and Na⁺ act at a common site (or whether either ion acts at more than one site) is not known, although Ni²⁺ did inhibit histamine-induced inositol phosphate formation in intact HeLa cells, implying an extracellular site of action (Arias-Montaña & Young, 1993b). The evidence from studies with Na⁺ and α_2 -receptor-mediated responses in platelets is that it is intracellular Na⁺ which is important (Motulsky & Insel, 1983). There is some evidence that Na⁺ slows the inactivation of dopamine D₂-receptors by N-ethylmaleimide (Neve, 1991) but the sites of action of Na⁺ and N-ethylmaleimide on histamine inhibition of [³H]-mepyramine binding to guinea-pig cerebellar membranes are clearly distinct (Yeramian *et al.*, 1985). Dithiothreitol, which increased the efficacy of H₁-agonist-mediated biochemical responses (Donaldson & Hill, 1986a) and potentiates H₁-agonist inhibition of [³H]-mepyramine binding (Donaldson & Hill, 1986b) in guinea-pig brain preparations, also remains effective in the presence of Na⁺ (K.W. Young & J.M.Y., unpublished observations). There is no indication that either N-ethylmaleimide or dithiothreitol affect H₁-antagonist binding, but the mechanism of action of both agents could be to promote a conformational change induced by agonist binding, without themselves causing such a change. Just how Na⁺ + Tris HCl might distinguish between agonist and antagonist binding, if this is established to be the case, remains to be determined.

For the majority of the antagonists surveyed, the effect of Na^+ was to decrease their affinity for the histamine H_1 -receptor. This is not surprising, given that most of the compounds we examined were developed as antagonists at the unperturbed receptor. It is fortunate for the accuracy of the assay that the affinity of [^3H]-mepyramine itself is only modestly reduced by 100 mM NaCl, i.e., that there is not a large inhibition of [^3H]-mepyramine binding by Na^+ and that the affinity of temelastine, used to define H_1 -receptor binding in saturation binding experiments, is effectively unaltered. There is no very clear relationship between antagonist structure and the affinity change induced by Na^+ . The structures of a number of the antagonists examined are shown in Figure 6. On the basis of the greater effect of Na^+ on the binding of [^3H]-QMDP, a quaternary amine, than on the binding of [^3H]-mepyramine, a tertiary amine, we suggested that the presence of the permanent positive charge might be an important factor (Treherne *et al.*, 1991). However, the

effect of Na^+ on the binding of norpirdonium and its quaternary derivative, pirdonium, is the reverse of what would have been predicted, since with this pair the shift of the inhibition curve for the tertiary amine is greater than with the permanently charged derivative. For only one of the compounds that we examined, the terfenadine metabolite, MDL 16,455A, was the affinity significantly increased in the presence of Na^+ . The effect is much smaller than the order of magnitude increase in the binding affinity of benzamide antagonists at the dopamine D_2 -receptor (Jarvie *et al.*, 1987; Neve, 1991), but it does suggest that it would be possible, at least in theory, to develop compounds selective for the conformation of the H_1 -receptor stabilized by Na^+ . Such a compound would be a useful pharmacological tool for an investigation of the functional significance of what appears to be a highly conserved mechanism for the regulation of agonist binding by intracellular Na^+ and H^+ .

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